SABiosciences™

BIOMOL GmbH
Waidmannstr. 35
22769 Hamburg
info@biomol.de
www.biomol.de
Phone: +49-40-8532600 or 0800-2466651 (D)
Fax: +49-40-85326022 or 0800-2466652 (D)

biomol

User Manual

SureSilencing™ siRNA

Genome-Wide High Purity Synthetic RNA Interference

See Purchaser Notification for limited use license and warranty information (pages 2 and 4).

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User Manual

(For Catalog Numbers SIH######X, SIM######X, SIR######X)

Ordering and Technical Service Contact Information:

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- Fax: 1-888-465-9859 (US) 301-682-7300 (outside US)
- On-line Order: www.sabiosciences.com
- E-MAIL: order@sabiosciences.com (to place an order)
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I. Background and Introduction

RNA Interference, a now commonplace and popular method for exploring gene function, suppresses the expression of a specific gene of interest in transformed mammalian cell culture or even in animal model. Upon suppression, missing or altered activities in the cell can be attributed to the function of the affected gene. The most commonly used technique, small interfering RNA (siRNA), is useful for many applications and has been proved to work optimally in many cells with high efficacies.
SureSilencing siRNA are designed using an experimentally validated algorithm. These synthetic double strands RNA specifically knock down the expression of specific genes by RNA interference. Each synthetic siRNA duplex is HPLC purified to provide the maximum amount of full length strand to target your gene of interest in full power.

Our experimentally verified siRNA design algorithm assures gene knockdown specificity and efficacy. An advanced specificity search in addition to BLAST built into the algorithm helps to reduce the potential off-target effects. At least two of the 4 provided SureSilencing siRNAs in each set is guaranteed to knock down the expression of target gene at the mRNA level by at least 70 percent in well transfected cells.

Benefits of the SureSilencing™ synthetic siRNA:

- **High Purity with low price:** All of our synthetic siRNA is HPLC purified that provides high quality siRNA to increase knockdown efficacy and lower off-target effects.
- **GUARANTEED!*:** Knock down the expression of any targeted human, mouse or rat gene by at least 70 percent with the full set order. Control for non-specific and off-target effects.
- **Convenient with customization opportunity:** People can order the full set or individual one for their preference. If customer has their own design in-hand, customized synthesis is also available.

* At least two of the four provided pre-designed SureSilencing™ siRNA are guaranteed to knock down the expression of target gene at mRNA level by at least 70 percent, as measured by real-time qRT-PCR in transfected cells as described in this User Manual. SureSilencing siRNA comes with a one-time–only replacement offer. If a set of SureSilencing siRNAs is ordered and less than 2 of the siRNAs result in gene silencing, SABiosciences will provide 4 additional siRNAs free of charge, once only. You will be asked to provide supporting data, demonstrating that the siRNA failed to knock down the target gene by at least 70% at the mRNA level under appropriate transfection conditions. Supporting data should include transfection efficiency data, quantitative silencing data, and data showing ≥ 70% knockdown of a positive control. This offer is valid for up to 6 months after the date of delivery.

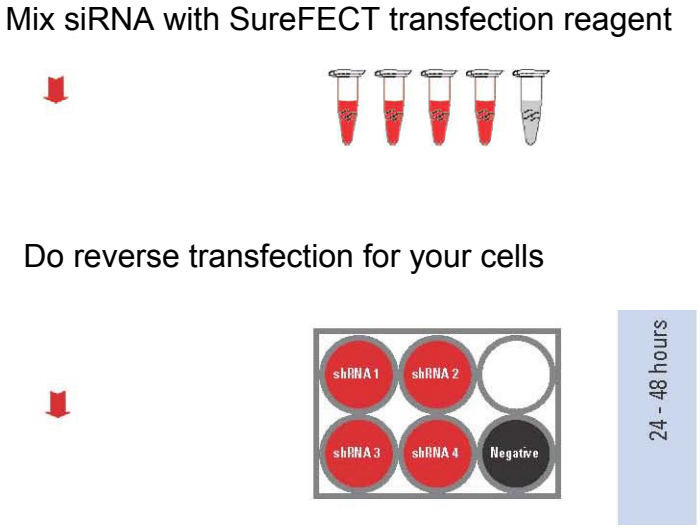
Mix siRNA with SureFECT transfection reagent
Do reverse transfection for your cells
shRNA 1
shRNA 2
shRNA 3
shRNA 4
Negative
24 - 48 hours
Isolate total RNA for checking knock down efficiency, or for your phenotype assay
Verify Suppression of Gene Expression
Using Real-Time PCR
Perform biochemical, cell, or molecular biological
assay of gene function

Figure 1: Overview of SureSilencing™ Synthetic siRNA Procedure.

II. Kit Contents and Related Information

A. siRNA (Set) Contents:

Component	Specification	Quantity
siRNA	SureSilencing™ siRNA, 12.5nmol in each tube	Four (4)
InfoSheet	SureSilencing siRNA InfoSheet with siRNA sequences	One (1)

B. siRNA (individual) Content:

Component	Specification	Quantity
siRNA	SureSilencing™ siRNA, 12.5 nmol in each tube	One (1)
InfoSheet	SureSilencing siRNA InfoSheet with siRNA sequences	One (1)

Resuspension Instruction:
Resuspend lyophilized siRNA duplex in 625 µl RNase-free ddH2O to get a 20 µM stock solution. Aliquots are recommended when repeated use is needed.

Storage Conditions:
All components included with this catalog number are shipped in ambient temperature. Store lyophilized siRNA duplex at -20 °C or below. Stable for at least 6 month from the date of shipment. Once resuspended, store at -20 °C or below in non-frost-free freezer, avoid contact with RNases and repeated freeze thaw cycles.

III. Additional Materials Required:

A. For Transfection:

We recommend using SureFECT™ Transfection Reagent (SABiosciences Cat. No. SA-01).

B. For Support Reagents

- siRNA Transfection Reagent: SureFECT™ Transfection Reagent SA-01
- Negative Control siRNA: SIH165860A
  SIM166769A
  SIR766773A
- RT-PCR Gene Specific Primer: Please check our website at: http://www.sabiosciences.com/RT2PCR.php
- RT-PCR House Keeping Gene Primer: Please check our website at: http://www.sabiosciences.com/QRTsearch.php?target=housekeeping

B. For Real-Time RT-PCR Verification of Gene Suppression:

1. RT² First Strand Kit Catalog Number C-03

2. SABiosciences RT^2 Real-Time™ SYBR Green PCR Master Mix
   Be sure to pick the correct one for the instrumentation in your laboratory.

RT^2 Real-Time™ SYBR Green / ROX: Specifically designed for:
All ABI and Stratagene Instrumentation
Eppendorf Mastercycler® ep *realplex* Instruments with ROX filter set

Catalog Number	Size
PA-012	For 2 RT^2*Profiler*™ PCR Arrays
PA-012-12	For 12 RT^2*Profiler*™ PCR Arrays
PA-012-24	For 24 RT^2*Profiler*™ PCR Arrays

RT^2 Real-Time™ SYBR Green / Fluorescein:
Specifically designed for BioRad iCylcer®, MyiQ®, and iQ5 Instrumentation

Catalog Number	Size
PA-011	For 2 RT^2*Profiler*™ PCR Arrays
PA-011-12	For 12 RT^2*Profiler*™ PCR Arrays
PA-011-24	For 24 RT^2*Profiler*™ PCR Arrays

RT^2 Real-Time™ SYBR Green:
Specifically designed for instrumentation that does not require a reference dye:
BioRad (MJ Research) Opticon, Opticon 2, and Chromo 4
Roche LightCycler® 480 System
Eppendorf Mastercycler® ep *realplex* Instruments without ROX filter set

Catalog Number	Size
PA-010	For 2 RT^2*Profiler*™ PCR Arrays
PA-010-12	For 12 RT^2*Profiler*™ PCR Arrays
PA-010-24	For 24 RT^2*Profiler*™ PCR Arrays

3. RT^2 PCR Primer Assay targeting the suppressed target gene of interest and a housekeeping gene, such as ACTB of GAPD, to normalize the real-time PCR results. Find the primer at: **http://www.sabiosciences.com/RT2PCR.php**
   PPH00073E-200: RT² PCR Primer Set for Human ACTB
   PPH00150E-200: RT² PCR Primer Set for Human GAPDH
   PPM02945A-200: RT² PCR Primer Set for Mouse Actb
   PPM02946E-200: RT² qPCR Primer Set for Mouse Gapdh
   PPR06570B-200: RT² PCR Primer Set for Rat Actb
   PPR06557A-200: RT² qPCR Primer Set for Rat Gapdh

4. RT² qPCR-Grade™ RNA Isolation Kit (SABiosciences Cat. No PA-001)

IV. Protocol:

A. Transfection:

We recommend the use of SureFECT transfection reagent (SABiosciences Cat. No. SA-01). For virtually all cell lines tested, SureFECT is an exceptional transfection reagent, providing maximal transfection efficiency and minimal toxicity.

If you have already optimized a transfection reagent and protocol for your cell line of interest, you may use that protocol to transfect the SureSilencing siRNA into the same cell line. Just be sure that the original protocol optimization used siRNA and assayed percentage of mRNA or protein knockdown or functional inhibition.

We recommend using reverse transfection protocols with the SureFECT transfection reagent. This is due to the time savings and improved reproducibility of using this method, compared to traditional forward transfection methods. SureFECT will also work well as a reagent for traditional forward transfection methods.

The following protocols are written on a **per well** basis, and are designed for the transfection of an adherent cell line, Hela (ATCC), with the SureFECT transfection reagent, using a 96-well cell culture plate. **We recommend that you set up three (3) replicate transfections for each of the four gene-specific siRNAs and the negative control SureSilencing siRNA using an optimized transfection protocol**.

Reverse Transfection Protocol

This is just a general guideline for 96 well transfection; the optimal conditions or amounts should be determined for each new cell line/cell type being transfected.

a. On the day of transfection, dilute 0.3 μl SureFECT with 37.7 μl Opti-MEM I medium for each well (the optimized condition for different cell line should be evaluated by customer).
b. Mix 2 pmol (2 μl) of each gene-specific siRNA and the negative control siRNA into 38 μl of the above Opti-MEM™ I diluted SureFECT (total volume = 40 μl). Mix gently. Prepare separate mixtures for each replicate.
c. Incubate 40 μl of the siRNA-SureFECT™ mixture in each well for 20 min at room temperature.
d. Add 160 μl cell suspension (prepared during the incubation step) at 8000~12000 cells/160 μl in regular culture media to each well of 96-well plate. Mix gently.
e. Incubate the cells at 37 °C in a CO_2 incubator for 24 to 72 hours.
f. Determine the target gene knock down at 48 hours (recommended) after transfection with real-time PCR.
g. Determine the phenotype change at the time point you optimized for your experiments.

Plating Format (wells/plate)	Surface Area (cm^2/well)	2 µM siRNA (µl)	SureFECT (µl)	Serum-free Medium (µl)	Total Plating volume (µl)	cell number per well
96	0.3	3	0.3	40	200	10000
24	2	9	1.5	100	600	50000
12	4	18	3	150	1200	1000000
6	10	36	6	200	2400	200000

Table 1. Recommended volumes for transfecting 30 nM* siRNA in various plating formats. SureFECT volumes and other conditions per well represent guidelines and may need to be optimized. *Note: 30 nM = 30 nmol/L = 30 pmol/mL = 30 fmol/µL

Traditional Forward Transfection Protocol

This is just a general guideline for 96 well transfection; the optimal conditions/amounts should be determined for each new cell line/cell type being transfected.

a. One day before transfection, seed 6000 cells in each well of 96-well plate with 200 µl of growth medium.
b. On the day of transfection, mix 2 pmol (2 µl) of each gene-specific siRNA or the negative control siRNA into 38 µl of the above Opti-MEM™ I Medium diluted SureFECT (total volume = 40 µl). Mix gently. Prepare separate mixtures for each replicate.
c. Incubate for 20 min at room temperature and add 40 µl of the siRNA-SureFECT™ mix to each well.
d. Add 160 µl of regular fresh media to each well. Mix gently.
e. Incubate the cells at 37 °C in a CO_2 incubator for 24 to 72 hours.
f. Determine the target gene knock down at 48 hours (recommended) after transfection with real-time PCR.
g. Determine the phenotype change at the time point you optimized for your experiments.

Our rigorous real-time RT-PCR protocol for verifying suppression by RNA interference relies on triplicate transfections for each gene-specific siRNA design and the negative control siRNA for statistically significant results. See the Appendix of the User Manual for more details.

Other siRNA delivery method protocols

For using other siRNA delivery methods such as electroporation, please refer the manufacturer's protocol for your cell line.

C. Assay Effects of Silencing Gene Expression

There are many ways to characterize the cellular effects caused by a decrease in the expression of a specific gene meditated by RNA Interference. The following is a brief list of possible ways. However, your experiments do not need to be limited by these suggestions.

Cells may be harvested, and RNA isolated for gene expression analysis using:

SABiosciences' RT^2*Profiler*™ PCR Arrays
SABiosciences' RT^2 Real-Time™ PCR Primer Sets and SYBR Green Master Mixes
SABiosciences' GEArray® Focused DNA Microarrays

Cells may be harvested, and protein isolated for:

SDS-PAGE and Western Verification
SABiosciences' Multi-Analyte Profiler ELISArray™ Kits
SABiosciences' Single Analyte ELISA Kits
Biochemical Assays

Cells may be left in wells or plates for:

SABiosciences' Cellular Activation Signaling ELISA (CASE™) Kits
Cell biological assays such as morphology and immunofluorescence staining

V. SureSilencing siRNA FAQs

What is the SureSilencing siRNA guarantee?

We guarantee that at least two of the set of four SureSilencing siRNA will knock down the expression of target gene by at least 70 percent in transfected cells as measured by real-time qRT-PCR relative to negative control siRNA-transfected cells using an optimized siRNA delivery condition. Please follow the recommendations in this User Manual to ensure the optimal level of knockdown. If more than two of the siRNAs in one set fail to knock down target gene expression, please contact our Technical Support to discuss your results and be prepared to provide your results in an Excel file as an email attachment. If a product failure is verified, we will send you another set of four pre-designed siRNAs for free.

What transfection method should I use with the SureSilencing siRNA?

We recommend using the SureFECT transfection reagent (SABiosciences Cat. No. SA-01). For the majority of cell lines tested, it is an exceptional reagent, providing superior combination of maximal transfection efficiency and minimal cytotoxicity. If you have a previously optimized siRNA transfection method for your cell line, you may use that method to transfect the SureSilencing siRNA. To quickly optimize transfection conditions, use a system that encodes an easily screening marker, such as GAPDH based enzyme activity assay. If your cells do not transfect well with lipid-based or chemical-based methods, electroporation method such as those provided by Lonza/Amaxa may be a useful alternative.

Can I use the SureSilencing siRNA with primary cells or macrophages or for injection into live animals?

Yes, there are many publications that have shown the efficient delivery of siRNA into primary cells, macrophages or other cell lines, and even animals with different methods. For these applications, we recommend finding relevant references describing proven methods before beginning your experiments.

VI. Troubleshooting Guide

A. Low transfection efficiency

1. Transfection efficiency primarily depends on the cell line used. Therefore, it is very important to optimize the transfection conditions for each cell type under study. Variable details to consider when optimizing the transfection conditions include cell density, cell viability, amount of siRNA, ratio of siRNA to transfection reagent, transfection complex formation time, and transfection incubation time (see the detailed protocols for our recommendations).

2. Use our positive control siRNA to optimize transfection efficiency.

3. If using the FAM labeled SureSilencing siRNA:
   Observe the cells carefully with fluorescence microscope under high power field. Check the transfected (FAM-positive) cells and the total number of cells (nuclear DNA stain) in the same fluorescent view of the same microscope field. Be sure to obtain numbers from several different randomly-chosen microscope fields in the interior (not toward the edges) of the cell culture well. The labeled siRNA signal is not as strong as EGFP signal you may ever see. The wash is necessary before observation in order to lower the background. Observations for long time may bleach the FAM signal.

NOTE: *DO NOT try to use the phase (cell) and fluorescence (GFP) views separately to estimate or count the total number of cells and the number of transfected cells, respectively.*

B. Knockdown not distinguishable by real-time PCR

1. **Poor transfection efficiency:**
   Make sure that your transfection efficiency is optimized. You must be looking at a population of cells that is nearly 100 percent transfected for an accurate determination of knockdown.

2. **Poor real-time PCR reproducibility:**
   Make sure that your triplicate real-time PCR threshold cycle values demonstrate a high degree of reproducibility with a standard deviation of roughly 0.25 to 0.33 cycles. A seventy (70) percent knockdown of expression will be observed as only a 1.74 difference in normalized C_t values for negative control vs. gene-specific siRNA transfected cells. This specific level of reproducibility is required to reliably detect such a difference.

3. **Low level of expression of gene of interest:**
   To accurately determine knockdown by real-time PCR, the level of expression of the gene of interest in control or un-transfected cells should be at least reasonably well expressed with a C_t value less than 30. Real-time PCR cannot determine the relative expression of genes expressed at a lower level (C_t > 30) with enough reproducibility to detect a seventy (70) percent knockdown of expression. Try using more input RNA (up to 5 μg) in the reverse transcription reaction and only dilute the

completed reaction by four-fold, adding 60 instead of 100 µl of ddH_2O, but still use 10 µl of the dilution to setup the PCR.

4. **Real-time PCR analysis not performed properly:**
   Make sure that real-time PCR analysis was set up and performed properly. Consult the Troubleshooting Guide of the RT^2 Gene Expression Assay User Manual if using the RT^2 PCR Primer Sets and RT^2 Real-Time™ SYBR Green PCR Master Mixes from SABiosciences. If using other reagents for real-time PCR, consult the original manufacturers' recommendations and suggestions.

Note: *Only use real-time PCR to determine the level of knock-down. No other RNA detection method (e.g. Northern Blot or conventional PCR) will be quantitative enough to observe a 70 percent knockdown. Western Blot is also unreliable because the success of knockdown at the protein level also depends on the quality of the antibody and the biological half-life of the protein, whereas RNA interference specifically acts at the RNA level. (See below.)*

C. The expression at RNA level is decreased but no decrease is observed at the level of protein or biochemical assay

The RT-PCR verification will confirm that expression has been decreased at the level of messenger RNA and that the SureSilencing siRNA functioned correctly. A change in the RNA level for a particular gene does not necessarily immediately correlate with a change in the amount of protein in the cell. If the protein has a long half-life, protein level change will take much longer to occur than RNA level change.

Appendix. Real-time RT-PCR Protocol for Verifying Suppression

A detailed description of the theory behind RNA interference validation using real-time PCR may be found in our white paper entitled:

"Did Your RNAi Experiment Work?!
Reliably Validating RNA Interference with Real-Time PCR"

For statistically significant results, the method relies on triplicate transfections for each gene-specific siRNA design and the negative control siRNA. It also requires triplicate real-time PCR reactions to characterize the targeted gene of interest (GOI) and a housekeeping gene (HKG) to normalize the results using the total RNA sample from each transfection. (Typical housekeeping genes include β-actin and GAPDH.)

Table 1: Setting up real-time PCR validation of suppression

The triplicate reactions for each gene in all five triplicate transfections may be conveniently setup in a 96-well PCR plate as depicted in this table. The table represents a 96-well plate. The reactions in the first set of six numbered columns will characterize expression of the GOI in the indicated RNA samples, while the second set of six numbered columns will characterize expression of the HKG in the corresponding RNA samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Design1 Txn1 P1 P2 P3			NC Design Txn3 P1 P2 P3			Design1 Txn1 P1 P2 P3			NC Design Txn3 P1 P2 P3		
B	Design1 Txn2 P1 P2 P3			Design3 Txn1 P1 P2 P3			Design1 Txn2 P1 P2 P3			Design3 Txn1 P1 P2 P3		
C	Design1 Txn3 P1 P2 P3			Design3 Txn2 P1 P2 P3			Design1 Txn3 P1 P2 P3			Design3 Txn2 P1 P2 P3		
D	Design2 Txn1 P1 P2 P3			Design3 Txn3 P1 P2 P3			Design2 Txn1 P1 P2 P3			Design3 Txn3 P1 P2 P3		
E	Design2 Txn2 P1 P2 P3			Design4 Txn1 P1 P2 P3			Design2 Txn2 P1 P2 P3			Design4 Txn1 P1 P2 P3		
F	Design2 Txn3 P1 P2 P3			Design4 Txn2 P1 P2 P3			Design2 Txn3 P1 P2 P3			Design4 Txn2 P1 P2 P3		
G	NC Design Txn1 P1 P2 P3			Design4 Txn3 P1 P2 P3			NC Design Txn1 P1 P2 P3			Design4 Txn3 P1 P2 P3		
H	NC Design Txn2 P1 P2 P3			BLANK			NC Design Txn2 P1 P2 P3			BLANK		
	Samples in column 1 to 6 are amplified using PCR primers specific for the GOI.						**Samples in column 7 to 12 are amplified using PCR primers specific for the HKG.**					

Unless otherwise indicated, follow the protocols described in the RT^2 Gene Expression Assays User Manual included with the SABiosciences RT^2 PCR Primer Sets

1. **RNA Isolation**

   a. Isolate total RNA from each of the 15 transfections.
   b. For cultured cells, use the RT² qPCR-Grade™ RNA Isolation Kit (PA-001). Be sure to include the recommended DNase treatment step.
   c. Also, make sure to perform the RNA quality control described in the RT^2 Gene Expression Assays User Manual.

2. **Reverse Transcription (First Strand cDNA Template Synthesis)**

   a. Perform one reverse transfection for each of the 15 total RNA samples (one per transfection).
   a. Follow the instructions in the RT^2 Gene Expression Assays User Manual included with the RT^2 PCR Primer Sets.
   b. For convenient pipetting below, dilute each completed 20-μl RT reaction 50-fold by adding 980 μl of ddH_2O.

3. **Primer Set and Master Mix Cocktail**

For real-time PCR, prepare two separate cocktails, one for the GOI and one for the HKG, using the following recipe:

Component	Volume
2X SABiosciences Real-Time PCR Master Mix	625 μl
ddH_2O	75 μl
RT² primer set for GOI or HKG	50 μl
Final Volume	**750 μl**

4. **Set Up the Reactions**
   Add 15 μl of the appropriate primer set and master mix cocktail and 10 μl of the appropriate diluted cDNA template (RT reaction) to the appropriate PCR wells as outlined in Table 1.

5. **Perform Real-Time PCR**
   Perform PCR as described in RT^2 Gene Expression Assays User Manual included with the RT^2 PCR Primer Sets.

6. Data Analysis:

An Excel-based data analysis template that automatically performs the calculations below is available for download from our website at the following address:

http://www.sabiosciences.com/rnaidataanalysis.php

a. Separately determine the average of the technical triplicate PCR C_t values and their standard deviations for both genes in each of the replicate transfections of each design and the negative control.

b. Separately calculate individual ΔC_t values for each biological replicate transfection of each design and the negative control:

GOI-Specific shRNA ΔC_t =
AVG GOI-Specific shRNA C_t (GOI) – AVG GOI-Specific shRNA C_t (HKG)

GOI-Specific shRNA ΔC_t STDEV = $\sqrt{STDEV_{GOI}^2 + STDEV_{HKG}^2}$

Negative Control shRNA ΔC_t =
AVG Negative Control shRNA C_t (GOI) – AVG Negative Control shRNA C_t (HKG)

Negative Control shRNA ΔC_t STDEV = $\sqrt{STDEV_{GOI}^2 + STDEV_{HKG}^2}$

c. Calculate the average ΔC_t and its standard deviation across the biological replicates for each design.

d. Calculate the average $\Delta\Delta C_t$ and its standard deviation for each design:

$\Delta\Delta$ C_t = Gene-Specific shRNA ΔC_t - Negative Control shRNA ΔC_t

$\Delta\Delta$ C_t STDEV = $\sqrt{STDEV_{Gene-Specific\ \Delta Ct}^2 + STDEV_{NegativeControl\ \Delta Ct}^2}$

e. Calculate the average knockdown and its 95 % confidence interval:

Percent Knockdown = 100 – (100 x 2^ (-$\Delta\Delta C_t$))
Lower 95 % Confidence Interval Boundary = 100 – (100 $\times$ 2^ (- $\Delta\Delta C_t$ + $\Delta\Delta$ C_t STDEV))
Upper 95 % Confidence Interval Boundary = 100 – (100 $\times$ 2^ (- $\Delta\Delta C_t$ - $\Delta\Delta$ C_t STDEV))

f. Interpretation

Successful Design: Observed KD $\geq$ 70 % and an upper 95 % C.I. boundary $\geq$ 55.5 %
Failed Design: Observed KD < 33.3 and a lower 95 % C.I. boundary < 55.5 %

Two out of the four designs should be successful. Use at least two of the four pre-designed siRNAs that demonstrate the greatest percent knock-down in your subsequent gene function assays and studies.

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Focus on Your Pathway™

BIOMOL GmbH
Waidmannstr. 35
22769 Hamburg
info@biomol.de
www.biomol.de
Phone: +49-40-8532600 or 0800-2466651 (D)
Fax: +49-40-85326022 or 0800-2466652 (D)

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